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E2F and GATA switches turn off WD repeat domain 77 expression in differentiating cells

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Abstract

WD repeat domain 77 (WDR77) is expressed during earlier lung development when cells are rapidly proliferating and absent in adult lung. It is re-activated during lung tumorigenesis and is essential for lung cancer cell proliferation. Signaling pathways/molecules that control *WDR77* gene expression are unknown. Promoter mapping, gel shift assay, and chromatin immunoprecipitation revealed that the *WDR77* promoter contains bona fide response elements for E2F and GATA transcriptional factors as demonstrated in prostate cancer, lung cancer and erythroid cells as well as in mouse lung tissues. The *WDR77* promoter is transactivated by E2F1, E2F3, GATA2, and GATA6 but suppressed by E2F6, GATA1 and GATA3 in prostate cancer PC3 cells. WDR77 expression is associated with the E2F1, E2F3, GATA2, and GATA6 occupancy on the *WDR77* gene and while in contrast the E2F6, GATA2, and GATA3 occupancy is associated with the loss of WDR77 expression during the erythroid maturation and lung development. More importantly, the loss of WDR77 expression that resulted from E2F and GATA switches is required for cellular differentiation of erythroid and lung epithelial cells. In contrast, lung cancer cells avoid post-mitotic differentiation by sustaining WDR77 expression. Altogether, this work provides a novel molecular mechanism by which *WDR77* is regulated during erythroid and lung development and lung tumorigenesis.

Introduction

The WD repeat domain 77 (WDR77) protein is composed of 342 amino acid residues and 7 putative WD-40 repeats and was identified as a component (MEP50) of the methylosome complex (1), a subunit (WD45) of the SMN complex (2), and a novel androgen receptor-interacting protein (p44) (3, 4). WDR77 localizes in the cytoplasm of epithelial cells and is required for cell proliferation at the growth stage of prostate development (4–6). In contrast, in the adult prostate, WDR77 expression is decreased and the WDR77 protein is translocated into the nucleus to function as an androgen receptor cofactor to establish and maintain luminal epithelia in a growth-arrested fully differentiated state (the G1/G0 cell cycle phase). Thus, WDR77 regulates the proliferation and differentiation of prostate

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epithelial cells through its distinct subcellular localization. The increased WDR77 gene expression and WDR77 protein translocation from the nucleus to the cytoplasm is associated with age-related prostatic intraepithelial hyperplasia and prostate tumorigenesis (4–6). The cytoplasmic WDR77 is also required for the proliferation of prostate cancer cells (4, 6, 7). Therefore, *WDR77* gene expression and WDR77 protein cytoplasmic translocation are critical events that lead to the proliferation of prostate epithelial cells and prostate tumorigenesis. More recently, we found that WDR77 is also highly expressed in the lung at the early development stage when cells are rapidly proliferating (8). In contrast, its expression is diminished in adult lungs when cells are fully differentiated. Loss of the *WDR77* gene expression led to growth arrest of lung epithelial cells at the G1 cell cycle phase. Thus, WDR77 also exerts an important role in growth of epithelial cells during lung development. More importantly, WDR77 was re-expressed in lung cancers and the shRNA-mediated silencing of WDR77 expression strongly inhibited growth of lung cancer cells in tissue culture and abolished growth of lung tumor xenografts in nude mouse (8, 9). These results reveal a novel role of WDR77 in the growth of lung epithelial cells as well as lung cancer. However, the molecular mechanisms that control WDR77 expression during development and tumorigenesis are unknown.

E2Fs are a group of genes encoding a family of transcription factors with E2F1, E2F2 and E2F3a as activators, and E2F3b and E2F4-8 as repressors (10–12). It was observed that E2F transcription factors switch from activators in proliferating progenitor cells to repressors in differentiating cells (13, 14). The discovery of its connection with pRB, and therefore cancer, greatly increased efforts aimed towards understanding how E2F controls cell proliferation (10, 15). Many DNA replication and cell cycle control genes are regulated by E2Fs (11). E2F family members are capable of transactivating E2F-responsive promoters and pushing immortalized quiescent cells into the S phase of the cell cycle (16–18). Numerous evidences show that deregulation of E2F activity plays a key role in tumorigenesis (19, 20). GATA factors are zinc finger DNA-binding proteins that control the development of diverse tissues by activating or repressing transcription of target genes (21). GATA1-3 are termed the hematopoietic GATA factors, based on their important activities to control distinct and overlapping aspects of hematopoiesis (22). GATA4-6 are expressed in endoderm- and mesoderm-derived tissues and control critical biological processes in the heart and lung (23). GATA2 maintains hematopoietic stem and progenitor cells, and in contrast GATA1 is essential for terminal differentiation of a subset of hematopoietic cells (24). Studies have shown that GATA2 binding to key *cis*-regulatory elements in proliferating progenitors is displaced by GATA1 as differentiation progresses (25), a process termed a GATA switch (26, 27). GATA switches are widely utilized developmental control tools (28).

By mapping *cis*-elements in the *WDR77* proximal promoter region and analyzing transcription factors targeting these elements, we found that E2F and GATA transcriptional factors regulate *WDR77* gene expression through E2F- and GATA-DNA binding sites in the *WDR77* promoter. E2F and GATA switches at the *WDR77* gene locus were observed and found to be required for lung development and erythroid maturation. Our data identify the WDR77 gene as a novel transcriptional target of E2F and GATA transcription factors, which might be involved in a wide range of biological processes such as development and cancer.

Materials and methods

Cloning and analysis of the proximal promoter region of the mouse WDR77 gene

A 1,284 bp of 5′-promoter region of the mouse *WDR77* gene was subcloned into pGL3-basic vector from a genomic clone containing the *WDR77* gene locus as described previously (4). To generate promoter deletions, two primers were designed and synthesized for PCR reactions and the resulted DNA fragments were subcloned into pGL3-basic vector to generate the luciferase reporter constructs. The point mutations were generated using the QuikChange Site-directed Mutagenesis kit (Stratagene). All DNA constructs were confirmed by DNA sequencing analysis. The pcDNA-E2F1, pcDNA-E2F6, pcDNA-GATA1, pcDNA-GATA2, pcDNA-GATA3, and pcDNA-GATA6 expressing constructs were constructed by subcloning the human E2F1, E2F6 GATA1, GATA2, GATA3, and GATA6 cDNAs into the pcDNA3.1 vector. Potential transcription factor binding elements in the promoter region were searched using TFBIND Program (http://tfbind.hgc.jp/). Alignments of human, rat and mouse *WDR77* promoter sequences were performed using the MacVector program.

Cell culture and cell growth analysis

PC3 and A549 cells were obtained from Dr. Isaiah J. Fidler at MD Anderson Cancer Center. PC3 cells were cultured in RPMI1640 medium supplemented with 5% fetal bovine serum (FBS). A549 cells were cultured in DMEM medium supplemented with 10% FBS, non-essential amino acids, vitamins, and sodium pyruvate. G1E-ER4 cells were a generous gift from M.J. Weiss (Children's Hospital, Philadelphia, PA) and were induced to differentiate with 10^{-7} M β-estradiol (Sigmal-Aldrich) as described (29).

For a cell growth assay, cells were plated on 24-well plates (5,000 cells/well) and counted every day. For a BrdU incorporation assay, cells (50–70% confluent) were placed on coverslips and cultured with 10 μM BrdU (Sigma-Aldrich, St. Louis, MO) for 4 hrs. The BrdU-labeled cells were detected using staining with a monoclonal anti-BrdU antibody (BD, Franklin Lakes, NJ).

Ectopic expression of WDR77

Mouse *WDR77* cDNA was subcloned into the expression lentivector pCDH (System Biosciences). Lentivirus production and transduction were performed as described previously (8). Three days after infection, cells were harvested for Western blot analysis and used for other experiments.

Transient transfection and luciferase assay

Cells were plated in 24-well plates (1.6×10^4 cells per well). Twenty-four hours later, the WDR77 promoter reporter (100 ng) and pRL-CMV (5 ng) were transfected using lipofectamine 2000 (Life Technologies). Three independent transfection mixtures were prepared for each plasmid construct and used to transfect cells in three individual wells. Forty-eight hours after transfection, cells were lysed and luciferase activity was measured by using the luciferase assay kit (Promega) according to the manufacturer's protocol. Values were expressed as mean ± standard deviations from triplicate experiments.

Western blot analysis

Whole protein lysates were prepared using the Passive Lysis Buffer (Promega) and resolved by 10% SDS-PAGE. Protein concentrations were determined using the Bradford Protein Assay Reagent (Bio-Rad). Western blotting detection was performed using primary antibodies against E2F1 (Santa-Cruz Biotechnology), E2F3 (Santa-Cruz Biotechnology), E2F6 (Santa-Cruz Biotechnology), WDR77 (30), or actin (Sigma). Immunoreactive proteins were detected using an enhanced chemiluminescence detection system (GE Healthcare) per the manufacturer's instructions.

Gel-shift assay

Gel shift assay was performed as previously described (31). Probes were prepared by annealing two oligos that contained a potential E2F binding site and labled by T4 polynucleotide kinase and γ-p^{32}-ATP. The human E2F1 cDNA was subcloned into pET15b vector (Novagen) and expressed in bacteria as described (31). E2F1 protein was purified by a Ni-NTA agarose (Qiagen) column and eluted by 100 mM imidazole. The DNA-protein complex was resolved on non-denaturing polyacrylamide gel electrophoresis in 0.25X TBE at 150 V for 2 h. The gel was dried under vaccumn and exposed to an x-ray film.

RNA extraction and quantitative PCR

Total RNAs were isolated from cultured cells using TRIzol reagent (Life Technologies) and reverse transcribed into cDNA using the Reaction Ready First Strand cDNA Synthesis Kit (SuperArray Biosciences). The cDNA products were polymerase chain reaction (PCR)–amplified with the RT^2 Real-Time SYBR Green PCR master mix and the gene-specific primer sets (SuperArray Biosciences) using a SmartCycler II (Cepheid). The Smart Cycler software program (version 2.0C) was used to process and quantify raw data.

Chromatin immunoprecipitation

Mouse lungs were derived from C57BL6/J mice as described (8). The chromatin immunoprecipitation (ChIP) assay was performed using EZ $ChiP^{tm}$ Kit (Upstate) following the manufacturer's instruction. Cells (n=3) or lung tissues (n=3) (sliced into 1 mm^3 pieces) were crosslinked with 10% formaldehyde for 5 min at room temperature and sonicated for total 2–3 min per sample using Brason Sonifer 250 at the setting of 10% output, 20-sec burst followed by 1–2 min cooling on ice. ChIP assay was performed with ant-E2F1, -E2F3, -E2F6, -GATA1, -GAT2, GATA3, or –GATA6 antibodies. Normal rabbit IgG was used as a negative control. Purified DNA products from chromatin immunoprecipitation were analyszed by quantitative PCR (Q-PCR) using primers specific for the *WDR77* promoter. All quantitative PCR signals were normalized to that of respective input samples. Values shown are the means and standard deviations of the results from 3 independent genomic preparations.

Isolation and culture of mouse lung epithelial cells

H-2K^b-tsA58 mice (n=5) were killed at the age of 21 days. The whole lung were then microdissected, minced and incubated with collagenase, pronase, and DNAse I at 37 oC for 0.5 hr. The epithelial organoids were separated from the stromal cells and debris by iso-

osmotic Percoll gradient centrifugation (500 × g, 30 min). After centrifugation, the stromal cells remained near the top of the gradient while the epithelial cell organoids banded with a higher density near the middle (ρ=1.05–1.07 g/ml) of the gradient. Epithelial organoids were collected and washed with M199 medium. The epithelial organoids were plated onto a culture dish that had been coated with 5 μg/ml of mouse laminin (Sigma-Aldrich) in keratocyte-SFM (GIBCO) supplemented with 2% fetal bovine serum (FBS), epidermal growth factor (EGF), and bovine pituitary extract. Upon reaching confluence, cells were re-plated to a new collagen-coated plate at a split-ratio of 1:2.

Immunohistochemistry

Lung tumor samples were described previously (8). Lung sections were blocked with 1% fish gel and incubated with a rabbit polyclonal anti-E2F1 (1:500) or -WDR77 (1:1,000) antibody overnight at 4°C. A streptavidin-biotin peroxidase detection system for use with prostate tissues (DAKO A/S, Grostrup, Denmark) was used according to the manufacturer's instructions; 3, 3′-Diaminobenzidine was used as the substrate. Cultured cells were allowed to grow on chamber slides and fixed with cold methanol (−20°C) for 10 min. Nonspecific proteins were blocked with 4% fish gelatin in PBS for 20 min. The cells were incubated with the primary antibodies described above at 4°C overnight and then incubated with goat anti-rat Alexa 595 (1:500; Invitrogen) at room temperature for 1 h. The cells were then washed with PBS, counterstained with SYTOX Green (Molecular Probes) for 10 min at room temperature, and mounted in Histogel (Linaris Histogel). A fluorescence confocal microscope was used to analyze the cells directly.

Statistical analysis

The protein bands were scanned using a densitometer, and the relative intensities were quantified using the ImageJ software program (ImageJ64, National Institutes of Health). Data are presented as the means of three or more independent experiments ± the standard deviations. A 2-tailed unpaired Student t-test was used to determine whether differences between control and experimental samples were statistically significant. *P* values less than 0.05 were considered statistically significant. In figures, *, ** and *** represent $p<0.05$, $p<0.001$ and $p<0.0001$, respectively.

Results

Identification of transcriptional regulatory elements in the *WDR77* promoter

Although WDR77 expression is regulated during lung and prostate development as well as during lung and prostate tumorigenesis (8, 32), the molecular mechanism that drives WDR77 expression remains unknown. We therefore decided to examine the transcriptional regulation of WDR77 expression. We cloned a mouse genomic fragment (−1,284 to +78) of the *WDR77* gene and inserted it into the luciferase reporter vector (pGL3-basic). Transient transfection of this reporter construct into PC3 cells resulted in much stronger (173-fold) luciferase activity than transfection of the empty vector (pGL3-basic) (Fig. 1A). The deletion from the 5′ end to −954 slightly (1.8-fold) enhanced, whereas deletion to −894 slightly (2.0-fold) decreased the luciferase repoter activity. Further deletion up to −49 did not affect the reporter activity significantly (Fig. 1A). But further deletion to −19 resulted in

almost complete loss of the reporter activity, indicating that the major *cis*-elemnts localize in the region of −49 to −19.

Upon close inspection of the proximal promoter region (−49 to −19), we found a putative E2F-binding site (top strand, $^{-49}$ATTCTCGC^{-42}) that resembles the consensus sequence (TTTSGCGC) recognized by E2F family of transcription factors (Fig. 1B, 1C). By DNA sequence alignment of the proximal promoter region (−243 to +78) with the E2F consensus sequence, we observed an additional putative E2F-binding site (bottom strand, $^{-143}$CTTCTCGC^{-150}) (Fig. 1B, 1C). These sequences are conserved in mouse, rat and human *WDR77* promoters (Fig. 1D). To confirm the involvement of two putative E2F-binding sites in mediating transcriptional activity of the *WDR77* promoter, we introduced point mutations in these sites (Fig. 1B, underlined nucleotides in lowercase). Mutation on a single E2F-binding site (E2Fmt1 or E2Fmt2) did not significantly affect the reporter activity of the *WDR77* promoter (−186 to +78) but mutation on both E2F-binding sites (E2Fmt1+2) dramatically (7.8-fold) decreased the reporter activity (Fig. 2A).

The reporter activity from the *WDR77* promoter with the E2F-sites mutated (E2Fmt1+2) is higher (7.5-fold) than that from the empty vector (pGL3) (Fig. 2A), indicating other *cis*-elements present in the *WDR77* promoter (−186 to +78). By searching for transcription factor-binding sites, a putative STAT-binding site ($^{-26}$TTCCCTCGGA^{-17}) was revealed (Fig. 1C, 1B). Mutation on this site (STATmt; Fig. 1B) slightly (1.7-fold) reduced the reporter activity of the *WDR77* promoter (−186 to +78) (Fig. 2B). However, mutations on both the STAT binding site and the two E2F binding sites (STATmtE2Fmt1+2) more significantly (16.7-fold) reduced the promoter activity (Fig. 2B), suggesting that E2F and STAT binding sites are two major regulatory *cis*-elements in the region of −186 to +78 of the *WDR77* promoter.

However, mutation on the two E2F-binding sites in the longer *WDR77* promoter (−243 to +78) affected the reporter activity less (2.3-fold versus 7.8-fold) than in the shorter *WDR77* promoter (−186 to +78) (Fig. 2A), suggesting other regulatory *cis*-elements exist in the region −243 to −187. By examining the DNA sequence of this region, we found three putative GATA-binding sites (Fig. 1B, 1C). In addition to the mutation on the two E2F-binding sites, the promoter mutated on two (sites II and III) or on three putative GATA-binding sites (E2Fmt2GATAmt or E2Fmt3GATAmt) reduced the promoter activity by up to 7.2-fold (Fig. 2C). Similar results were obtained with lung cancer A549 and PC14 cells (Fig. S1). To further confirm the regulatory function of GATA on the *WDR77* promoter, the expression vectors encoding human GATA proteins were co-transfected with the E2F mutated *WDR77* promoter (−243/+78E2Fmt) reporter construct. Western blot analysis indicated that GATA1, GATA2, GATA3, and GATA6 were expressed at comparable levels (Fig. S2). Figure 2D shows that GATA2 and GATA6 enhanced but GATA1 and GATA3 inhibited the promoter activity (Fig. 2D).

These analyses identified 6 regulatory *cis*-elements recognized by three transcription factors (GATA, E2F, and STAT) in the region −243 to +78 of the *WDR77* promoter (Fig. 1B).

Regulation of the *WDR77* gene by E2F

We used a gel shift assay to test whether the identified putative E2F-binding sites directly interact with the E2F1 protein. Human E2F1 protein was expressed in and purified from bacteria (Fig. 3A) and interacted with the DNA probe containing E2F binding site I or II (Fig. 3B, lanes 3 and 6). Mutations on the conserved nucleotides (Fig. 1C) in the E2F probes abrogated the DNA-E2F1 complex formation (Fig. 3B, lanes 2 and 5), confirming the specificity of the observed interactions of the E2F1 protein with the E2F-binding sites in the *WDR77* promoter.

To further confirm the critical regulatory function of E2F1 on the *WDR77* promoter, the expression vector encoding human E2F1 proteins was co-transfected with the *WDR77* promoter (−243 to +78) reporter construct. Figure 3C shows that E2F1 enhanced the *WDR77* promoter activity in a dosage-dependent manner. The mutated construct [pGL3-WDR77(E2Fmt)], in which two E2F-biding sites were mutated, did not respond to ectopic expression of E2F1. Similar results were observed with E2F3a. In contrast, ectopic expression of E2F6, which functions as a transcriptional repressor, inhibited the *WDR77* promoter activity in a manner dependent on the presence of E2F-binding sites (Fig. 3C). Thus, E2F transcription factors regulate *WDR77* promoter activity through E2F-binding sites.

Many E2F downstream target genes are related to cell cycle progression and DNA synthesis and are regulated in a cell growth-dependent manner (11). As a consequence of the transcriptional control by E2F, *WDR77* gene expression might be regulated during cell cycle progression. To examine this possibility, we measured WDR77 mRNA and protein levels in A549 cells at various time points following cell cycle re-entry. As shown by the flow cytometry data (Fig. S3A), asynchronously growing cells arrested with a G1 DNA content by serum starvation (STV) and released to fresh medium containing hydroxyurea (HU) (0h), DNA content did not increase significantly, confirming that HU blocked DNA replication (33). After serum starvation and HU treatment, the majority (67.18%) of cells were arrested with a G1 DNA content, which first decreased (0–6h) and then increased (8–12h) following the block-release (Fig. S3A, B). In contrast, the S-phase cell population significantly increased 2h after the block-release and reached the maximum at 4h after the block release (Fig. S3A, B). As expected, E2F1 mRNA levels peaked at the transition from G1 to S, (0h after HU block-release) and decreased to the lowest levels observed 8h after HU block-release (Fig. S3C). THe WDR77 mRNA expression pattern matched perfectly with that of E2F1 mRNA (Fig. S3C), suggesting that E2F1 may regulate *WDR77* gene expression during cell cycle progression. Decreased E2F1 expression was associated with the tretinoin-induced differentiation of promyelocytic cells (Fig. S4A; GDS3089) and embryonic stem cell differentiation (Fig. S4B; GDS3729). WDR77 expression was also decreased during these differentiation progresses (Fig. S4A, S4B). E2F1 expression was up-regulated in lung hyperplasia (Fig. S4C, left panel) and as expected, WDR77 expression was also up-regulated during lung tumorigenesis (Fig. S4C, right panel). These observations suggest that WDR77 expression is associated with E2F1 during cell cycle progression, cell differentiation, and lung tumor initiation.

E2F and GATA switches turn off *WDR77* expression during erythroid maturation and lung development

The development of mature blood cells from progenitors and maintenance of the differentiated state is coordinated by GATA1 and GATA2 (34). This process involves the acquisition of tissue-specific functions and progressive restriction of proliferative potential, usually culminating in G1-phase arrest. G1E-ER4 cells were derived by targeted gene deletion of the *GATA1* gene and stably express a conditional, estrogen-activated form of GATA1 (GATA1 fused to the ligand binding domain of the estrogen receptor) (29, 34). G1E-ER4 cells proliferate continuously in culture as immature erythroblasts and undergo terminal maturation when GATA1 function is restored in the presence of estrogen. G1E-ER4 cells have proven to be a convenient and physiologically relevant system for studying various aspects of erythroid biology (35–37).

As reported (35–37), addition of estradiol triggered terminal maturation as indicated by hemoglobin expression (Fig. 4Aii; Fig. 4B; Fig. 6A) and proliferation arrest (Fig. 4C) at the G1 phase (Fig. S5). The terminal maturation is associated with the GATA expression switch from GATA2 to GATA1 (Fig. 5A). E2F1 and E2F3a expression was slightly decreased (Fig. 5B; 5C) during the maturation. In contrast, E2F6 expression was induced upon addition of estradiol (Fig. 5C). As expected, WDR77 expression at both mRNA (Fig. 5B) and protein (Fig. 5C, 5D) levels was significantly decreased during the maturation. To investigate how GATA and E2F factors interact directly with the *WDR77* gene *in vivo*, we performed ChIP experiments with control and estradiol-stimulated G1E-ER4 cells with antibodies against GATA and E2F factors. GATA2 and E2F1 occupancy on the *WDR77* promoter was decreased but GATA1 and E2F6 occupancy was increased during the maturation (Fig. 5E). These results support a model in which GATA and E2F switches from activators to repressors to turn off *WDR77* expression during the G1E-ER4 cell maturation.

We previously showed that *WDR77* was expressed at high levels at the earlier stages of mouse lung development but its expression was diminished in the adult mouse lung (8) (Fig. 6A, top panel, lane 4 versus lanes 1–3; Fig. S6). Western blot analysis revealed that E2F1 expression was also high at the earlier stages of lung development and its expression was significantly decreased in the adult mouse lung (Fig. 6A, 2nd panel, lane 4 versus lanes 1–3). Similarly, E2F3a expression was also decreased (Fig. 6A, 3nd panel, lane 4 versus lanes 1–3). In contrast, the repressive E2F factor (E2F6) was expressed only in the adult mouse lung (Fig. 6A, 4th panel, lane 4 versus lanes 1–3). These observations are consistent with mRNA expression patterns obtained with DNA microarray analysis (Fig. 6B). RT-PCR analysis indicated that expression of GATA3 (4.9-fold increase) and GATA6 (66.7-fold decrease) was also changed dramatically during the lung development (Fig. 6C). Consistently, ChIP assay indicated that E2F1, E2F3 and GATA6 occupancy on the *WDR77* gene was decreased but E2F6 and GATA3 occupancy was increased during mouse lung development (Fig. 6D; Fig. S7). Thus, GATA and E2F switches from activators to repressors may also play an important role to turn off WDR77 expression during mouse lung development.

WDR77 expression promotes cellular proliferation but inhibits cellular differentiation

By searching Gene Expression Omnibus (GEO), we found that decreased *WDR77* expression is associated with various cellular differentiation processes such as erythroid differentiation (Fig. S8A), erythropoirsis (Fig. S8B), cardiopoiesis (Fig. S9A), ES cells differentiating into embryoid bodies (Fig. S9B), and Schwann cell development (Fig. S9C). To test whether loss of *WDR77* expression has any functional consequence on cellular differentiation, we ectopically expressed the WDR77 protein in E1G-ER4 cells via the lenctivirus (Fig. 5D, lanes 3 and 4). Estradiol treatment for 30h decreased endogenous but not ectopic WDR77 expression (Fig. 5D, lane 4 versus lane 2). The estradiol-induced cell maturation (Fig. 4A, iv versus iii; Fig. 4B), growth arrest (Fig. 4C), and cell cycle arrest at the G1 phase (Fig. S6) were significantly inhibited by ectopic WDR77 expression. Our findings demonstrate that enforced WDR77 expression overrides cellular proliferation arrest and maturation. Together, these results indicate that the down-regulation of WDR77 expression by E2F and GATA switches is required for the mature erythroid phenotype.

A transgenic mouse strain (*H-2K^b-tsA58*) whose tissues harbor a temperature-sensitive simian virus 40 large tumor antigen (LTA) was used to isolate and propagate primary cells from different organs for long-term culture (38, 39). We used this mouse strain to generate primary cultures of mouse lung epithelial cells. We isolated epithelial cells from the lung and cultured them at a permissive temperature (33°C). At this temperature, LTA is expressed in lung epithelial cells and epithelial cells are immortal (Fig. 7A; 7C). Isolated lung epithelial (LEC-LTA^{ts}) cells expressed cytokeratine 18 (CK18), a general epithelial cell marker, and SP-C, a lung epithelial cell marker (Fig. 7A). These cells grew normally at 33°C (Fig. 7C) and highly proliferative (Fig. 10). Transferring LEC-LTA^{ts} cells from a permissive temperature (33°C) to a nonpermissive temperature (37°C) inactivated the LTA, resulting in increased CK18 and SP-C expression (Fig. 7A), cell growth arrest (Fig. 7C), and loss of proliferation (Fig. S10). WDR77 was highly expressed in LEC-LTA^{ts} cells cultured at 33°C (Fig. 7A, 7B); however, WDR77 expression was lost in LEC-LTA^{ts} cells cultured at 37°C (Fig. 7A, 7B). Lentivirus-mediated re-expression of WDR77 (Fig. 7B, lane 3) relieved the arrest of these cells' growth (Fig. 7C; Fig. S10) and cellular differentiation (Fig. 7A, left panels). Our tissues constrain cell multiplication by instructing cells to enter into post-mitotic, differentiated states. Loss of WDR77 expression may be an eseential step for such a process.

It is apparent that cancer cells use various strategies to avoid terminal differentiation to sustain proliferation (40). Re-expression of WDR77 has been observed in various cancers and is required for cancer cell proliferation (4, 8). WDR77 expression may be one strategy for cancer cells to avoid terminal differentiation. To test this possibility, we silenced *WDR77* in A549 cells as previously described (8) and then immunostained the differentiation markers (SP-C and CC-10) of lung epithelial cells. A549 cells expressed low levels of SP-C and CC-10 (Fig. 8I, 8VII) but silencing WDR77 significantly enhanced SP-C and CC-10 expression. This result suggests that WDR77 expression avoids lung cancer cell differentiation, which is similar to that observed for normal lung epithelial cells (Fig. 7).

Discussion

WDR77 was initially characterized as a transcriptional coregulator of ligand-activated androgen receptor, involved in the control of prostate functions (3, 30) and then was shown to play an essential role for proliferation of epithelial cells as well as cancer cells (8, 9, 41, 42). In the present study, we identified *WDR77* as a target gene of E2F and GATA transcription factors and WDR77 expression altered by E2F and GATA switches is required for growth arrest and cellular differentiation during development.

Based on promoter mapping analysis, *in vitro* DNA-protein interaction assay, transient transfection assay, and ChIP experiment, our data clearly demonstrate that E2F and GATA transcription factors regulate *WDR77* gene expression through binding to the proximal promoter region. Similar to many E2F downstream target genes (11), *WDR77* gene expression is also regulated during cell cycle progression and correlated with E2F1 expression during promyelocytic and stem cell differentiation. Given the essential role of E2F transcription factors in cell cycle progression and observed essential role of WDR77 in cellular proliferation at earlier stages of lung and prostate development (8, 9, 41, 42), WDR77 might be an important target of E2F to control cellular proliferation. WDR77 is re-expressed or over-expressed in lung and prostate cancer and required for proliferation of cancer cells (4, 8, 41). The E2F/pRB pathway is frequently deregulated in cancer by either pRB inactivation or by E2F1 overexpression (20). Supporting the idea that this pathway might contribute to WDR77 deregulation during carcinogenesis, we found that lung hyperplasia display increases in both E2F1 and WDR77 expression. Therefore, the identification of E2F1 as a transcriptional regulator of WDR77 significantly broadens our understanding of the mechanisms leading to WDR77 re-expression in cancer. The data present here open the possibility that the E2F switch (to the opposite direction) observed during lung development may also exist during lung tumorigenesis, which turns on *WDR77* gene expression to drive cellular proliferation. Our data provide the molecular basis for the observed WDR77 expression patterns during development and tumorigenesis.

Considerable progress has been made in elucidating GATA factor-dependent genetic networks that control blood cell development (43). GATA2 is required for the genesis and/or function of hematopoietic stem cells, whereas GATA1 drives the differentiation of hematopoietic progenitors into a subset of the blood cell lineages. GATA1 and GATA2 were known to be expressed reciprocally during erythropoiesis. The GATA switch model, in which GATA1 displaces GATA2 from chromatin sites to instigating distinct transcriptional output, was proposed to explain how GATA1 and GATA2 function distinctly through common sites to yield different biological activities (28). The GATA switch has been demonstrated in erythroid cells and megakaryocytes (25) as well as other cell types during cellular differentiation (28). E2F switching from activators (E2F1-3) to repressors (E2F4-8) is also associated with cellular differentiation (13). By promoter analysis, we found two E2F- and three GATA-binding sites in the *WDR77* promoter and demonstrated that the *WDR77* promoter responds distinctly to E2F and GATA factors. Further, WDR77 expression was decreased via the E2F and GATA switch during the erythroid differentiation and lung development.

During the development, a period of extensive proliferation is needed to generate the required numbers of progenitor cells for tissue and organ formation. This must be accompanied or closely followed by cell differentiation in order to generate the range of functional cells at the correct time and place. Transcriptional networks such as E2F and GATA orchestrate complex developmental processes to provide the intricate balance between proliferation and differentiation. Our previous studies demonstrated an essential role of WDR77 in cellular proliferation (4, 8, 41). Here, we showed that E2F and GATA transcriptional networks control WDR77 expression. In the proliferative cells, WDR77 expresses at high levels presumably driven by the active E2F and GATA transcriptional factors to promote proliferation. In contrast, WDR77 expression is diminished in the terminal differentiated cells caused by E2F and GATA switches. As a consequence, cellular proliferation stopped and terminal differentiation proceeded. This finding provides an intricate connection that contributes to precise coordination between proliferation and differentiation.

Cells respond to injury by modulating their phenotype from a differentiated, contractile state to a synthetic and proliferative phenotype (44). This de-differentiation is a critical aspect of many pathological processes (45), including cancer (46). Despite considerable efforts, the details of cell differentiation and de-differentiation in development and disease have remained elusive. We found that WDR77 expression is turned off during lung development via E2F and GATA switches, which is required for lung cell differentiation. However, WDR77 is re-expressed during lung tumorigenesis, which is essential for lung cancer cell proliferation (8). It has been notice that tumor cells use various strategies to avoid entrance into a differentiated and post-mitotic state (40); sustained WDR77 expression might be one of such strategies. The future study would be to further investigate whether the reverse-switches of E2F and GATA contribute to WDR77 re-expression in cancer cells to promote cell proliferation and avoid differentiation. This study would further advance our understanding of the complex mechanisms that regulate differentiation and phenotypic switching in disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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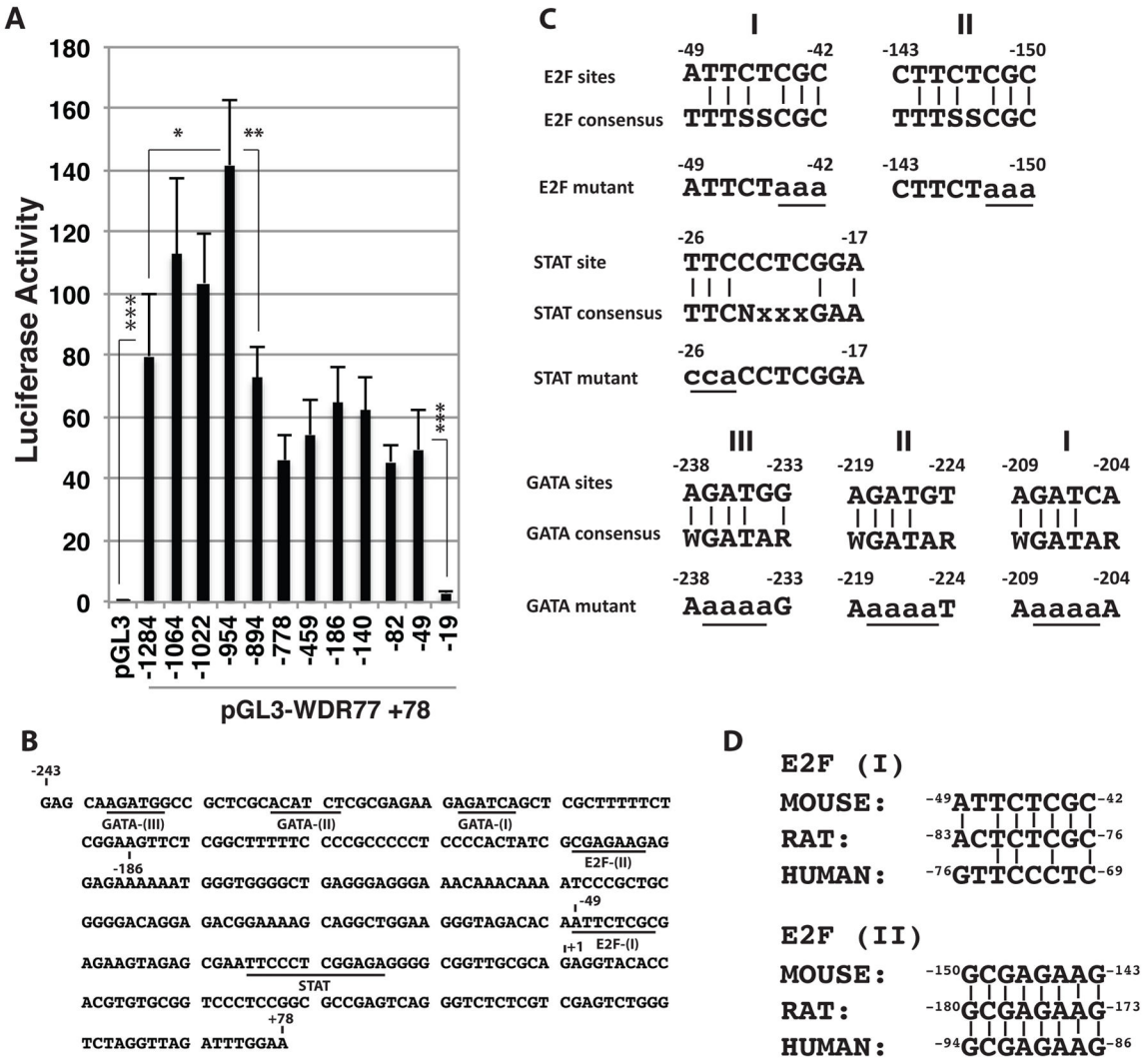

Figure 1.

Identification of *cis*-elements in the promoter of the *WDR77* gene. A. *WDR77* promoter luciferase assay. Various truncations of the *WDR77* promoter were subcloned into a luciferase reporter (pGL3) and submitted to luciferase assay. PC3 cells were transfected with 100 ng of luciferase reporter constructs containing truncations of the WDR77 promoter and 5 ng of a pR-LUC internal control plasmid. Transfected cells were allowed to grow for 2 days and harvested for the luciferase assay. The values represent the mean ± SD ($n = 3$). B. Sequence of the mouse *WDR77* gene promoter region (−243 to +78). Putative binding-sites of transcription factors are underlined. C. Alignment of putative E2F, GATA and STAT binding sites found in the mouse promoter with the consensus sequences. D. Alignment of putative E2F sites in mouse, rat, and human *WDR77* promoters.

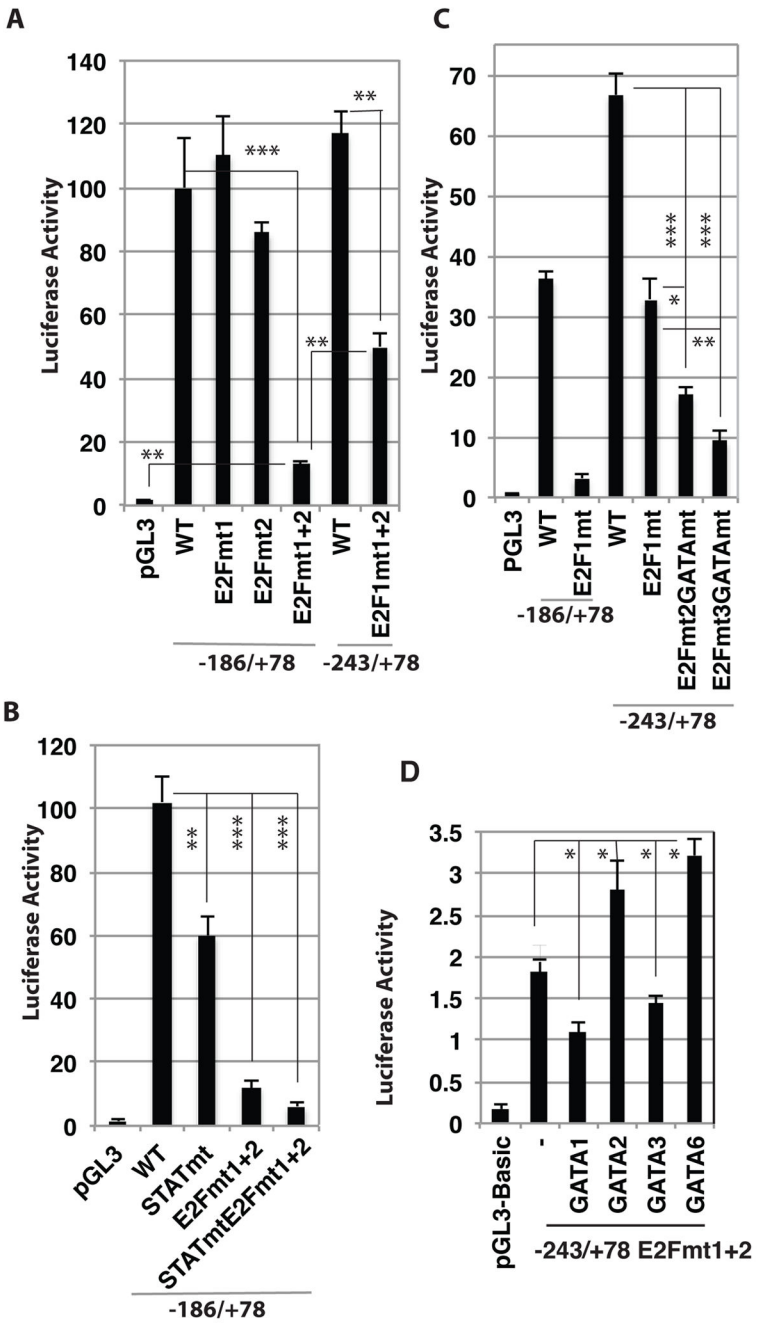

Figure 2.

Effects of the mutagenesis of the putative E2F (A), STAT (B), and GATA (C) binding sites in the *WDR77* promoter. Wild-type (WT) or mutated (mt) *WDR77* promoters were subcloned into a luciferase reporter (pGL3) and subjected to the luciferase assay. (D). Effects of GATA proteins on the *WDR77* promoter. PC3 cells were transiently transfected with the *WDR77* promoter reporter with the mutation at two E2F-binding sites (−243/+78E2Fmt1+2) (100 ng), pRL-CMV (5ng) and pLX304-GATA1, -GATA2, -GATA3 or -GATA6 (50 ng). The dua-luciferase assay was performed 48h post transfection.

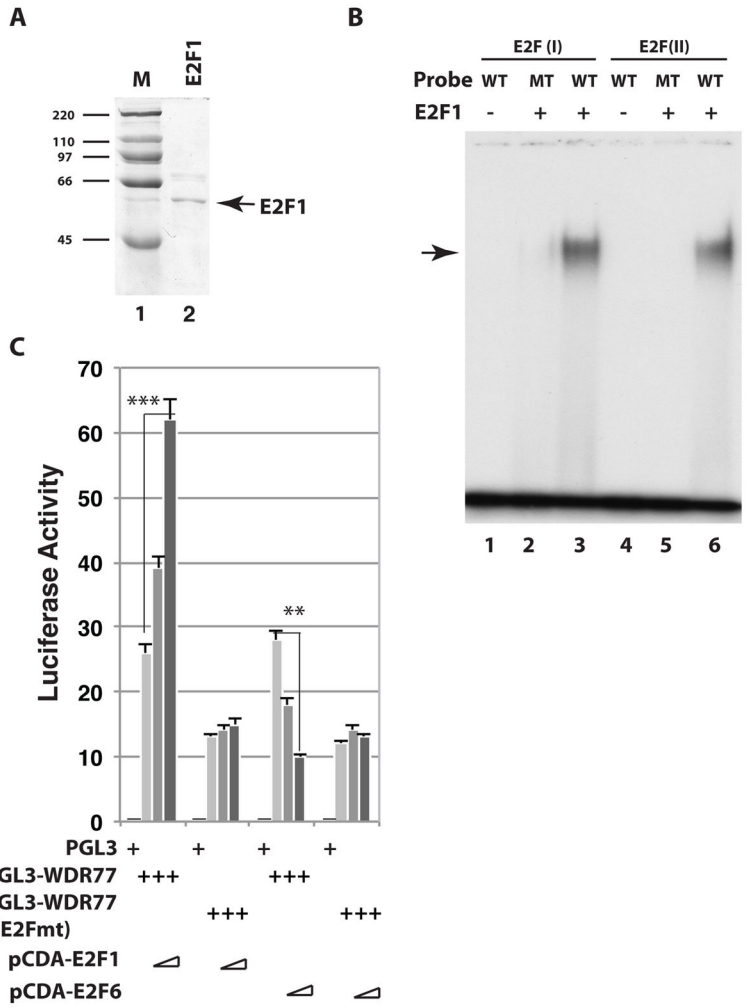

Figure 3.

E2F transcriptional factors regulate *WDR77* gene expression. A. SDS-PAGE of the recombinant human E2F1 protein. The purified E2F1 protein (100 ng) was loaded onto a 10% SDS-polyacrylamide gel and the gel was stained with Coomassie Brilliant Blue R250. B. Interaction of the E2F1 with the E2F sites of the *WDR77* promoter. Two fmoles wild-type (lanes 3 and 6) or mutant (lanes 2 and 5) E2F binding site I (lanes 1–3) or II (lanes 4–6) probes were incubated without (lanes 1 or 4) or with 2.5 ng (lanes 2, 3, 5 or 6) of the recombinant human E2F1 protein. The reaction mixtures were incubated at room temperature for 30 min and submitted for gel shift assay. C. PC3 cells were transiently transfected with the wild-type (WT) or two E2F sites-mutated (E2Fmt) at the *WDR77* promoter reporter (100 ng) together with expression vector (25 or 50 ng) for E2F1 or E2F6.

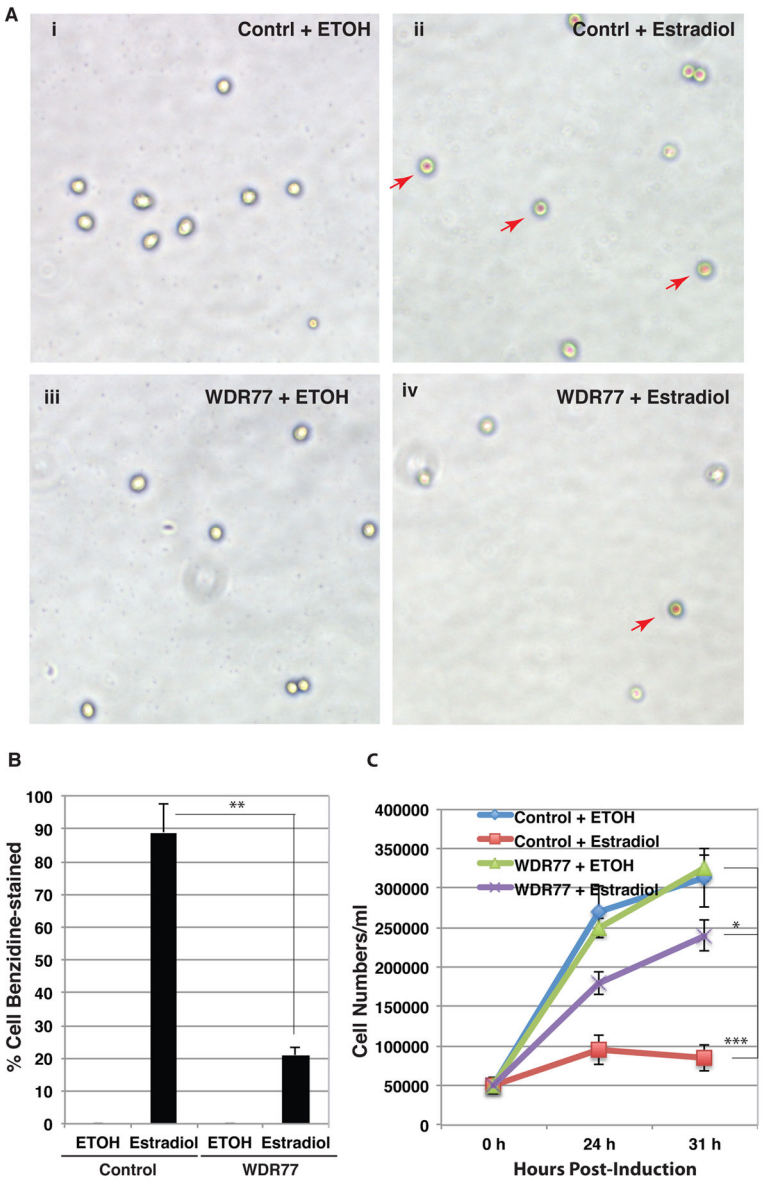

Figure 4.

WDR77 inhibited the maturation of G1E-ER4 cells. A. Benzidine staining of control (i, ii) and WDR77-expressing (iii, iv) G1E-ER4 cells before (i, iii) and after (ii. iv) estradiol-induced activation of GATA1. B. Percentage of cells stained positively by benzidine. C. Growth curves of control and WDR77-expressing G1E-ER4 cells before and after estradiol-induction.

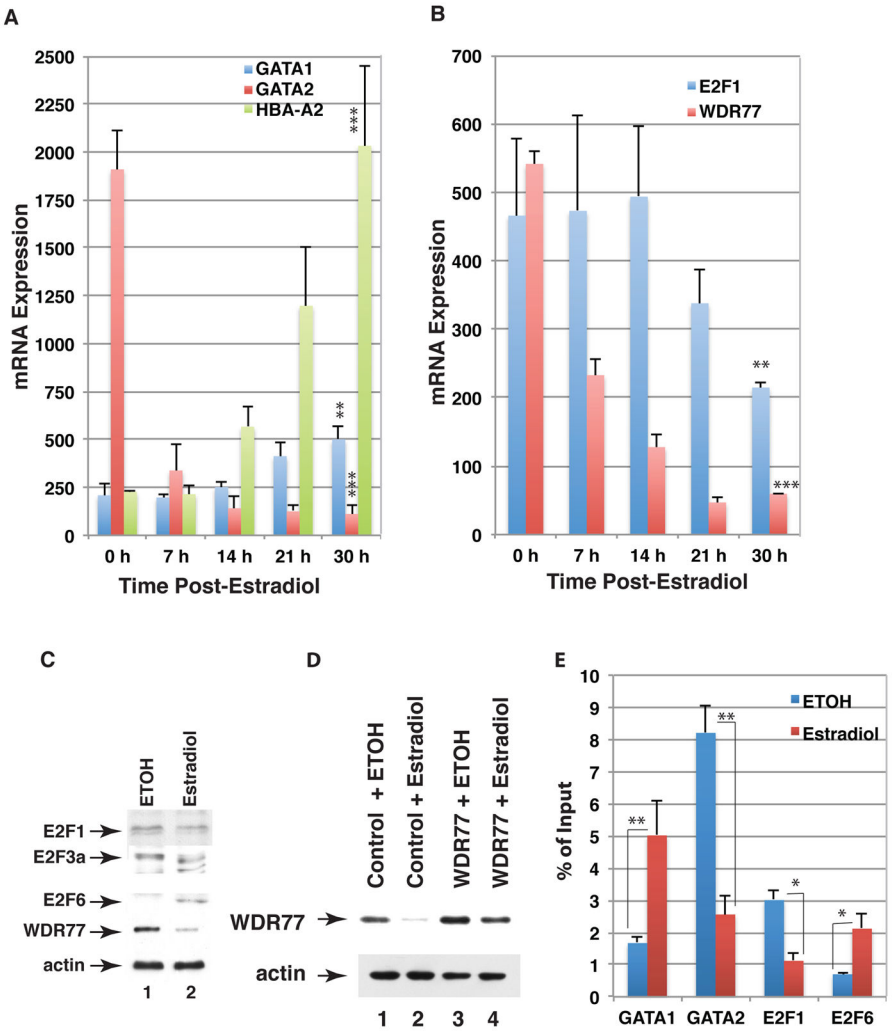

Figure 5.

GATA and E2F switches regulated WDR77 expression during erythroid maturation. A, B. Expression of GATA1, GATA2, hemoglobin alpha 2 (HBA-A2), E2F1, and WDR77 mRNA during erythroid maturation. Data was extracted from GDS568 (www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS568). C. Expression of E2F1, E2F3a, E2F6, WDR77, and actin during the maturation of G1E-ER4. Whole cell extracts were made from G1E-ER4 cells treated with ETOH (lane 1) or estradiol (lane 2) for 30 h and submitted for Western blot analysis with antibodies as indicated. D. Ectopic expression of WDR77 in G1E-ER4 cells. Whole cell extracts were made from G1E-ER4 cells infected with control (lanes 1 and 2) or WDR77-expressing (lanes 3 and 4) lentivirus treated with ETOH (lanes 1 and 3) or estradiol (lanes 2 and 4) for 30 h and submitted for Western blot analysis with antibodies as indicated. E. GATA1, GATA2, E2F1, and E2F6 were associated with the *WDR77* gene *in vivo*. ChIP assay using antibodies against GATA1, GATA2, E2F1, and E2F6 or control antibodies to immunoprecipitate chromatin from G1E-ER4 cells treated with ETOH or estradiol for 30 h. The average results of three separate experiments are shown.

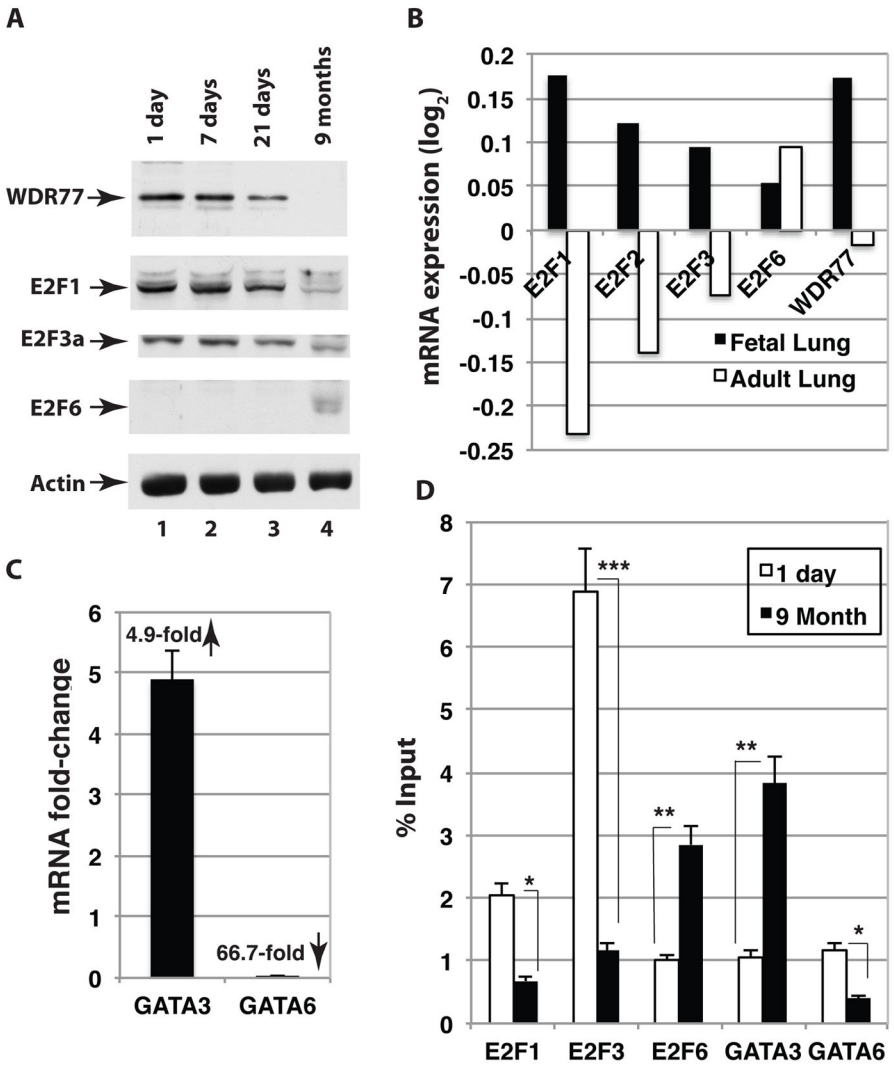

Figure 6.

GATA and E2F switches regulated WDR77 expression during lung development. A, B. Altered expression of E2F transcription factors and WDR77 during lung development. Western blot analysis of whole protein lysates made from lungs of mice at the ages of 1, 14, 30, and 270 days were performed with antibodies as indicated (A). The mRNA expression data (B) was extracted from GDS3834 (www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS3834). C. GATA expression during the lung development. Total RNAs were isolated from lungs of mouse at the ages of 1 day and 9 months and quantified by real-time quantitative PCR. The mRNA fold-change = mRNA expression in 9-months old mouse/ mRNA expression in 1 day mouse. D. The occupancy of E2F and GATA transcription factors on the *WDR77* gene during lung development. ChIP assay was performed with lungs derived from mice at the age of 1 and 270 days with anti-E2F1, -E2F3, –E2F6, -GATA3, or -GATA6 antibody.

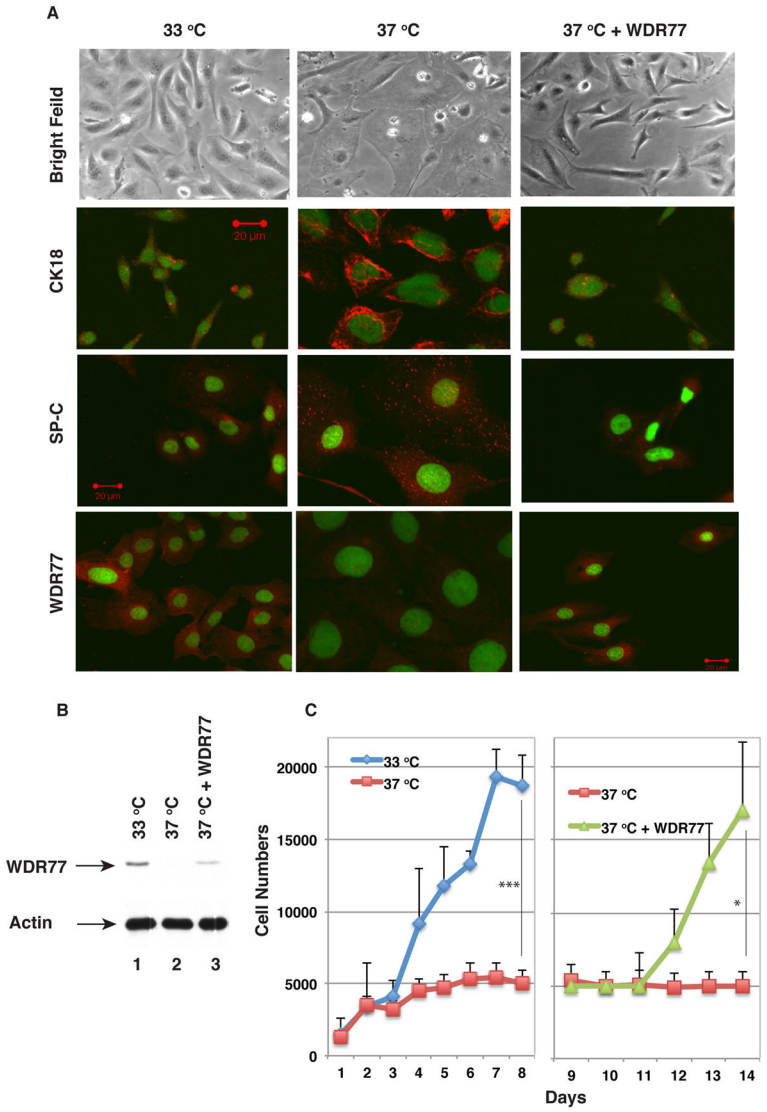

Figure 7.

WDR77 inhibited lung epithelial cell differentiation. A. Lung epithelial cell diffrentiation. Lung epithelial cells or WDR77-expressing lung epithelial cells were grown at 33 °C or 37 °C and immunostained for CK18, SP-C and WDR77. B. WDR77 expression was lost in differentiated lung epithelial cells. Western blot analysis of WDR77 expression in lung epithelial cells (lanes 1 and 2) or WDR77-expressing lung epithelial cells (lanes 3) grown at 33 °C (lane 1) or 37 °C (lanes 2 and 3). C. Ectopic expression of WDR77 promoted growth of lung epithelial cells. Growth curves of lung epithelial cells or WDR77-expressing lung epithelial cells grown at 33 °C or 37 °C.

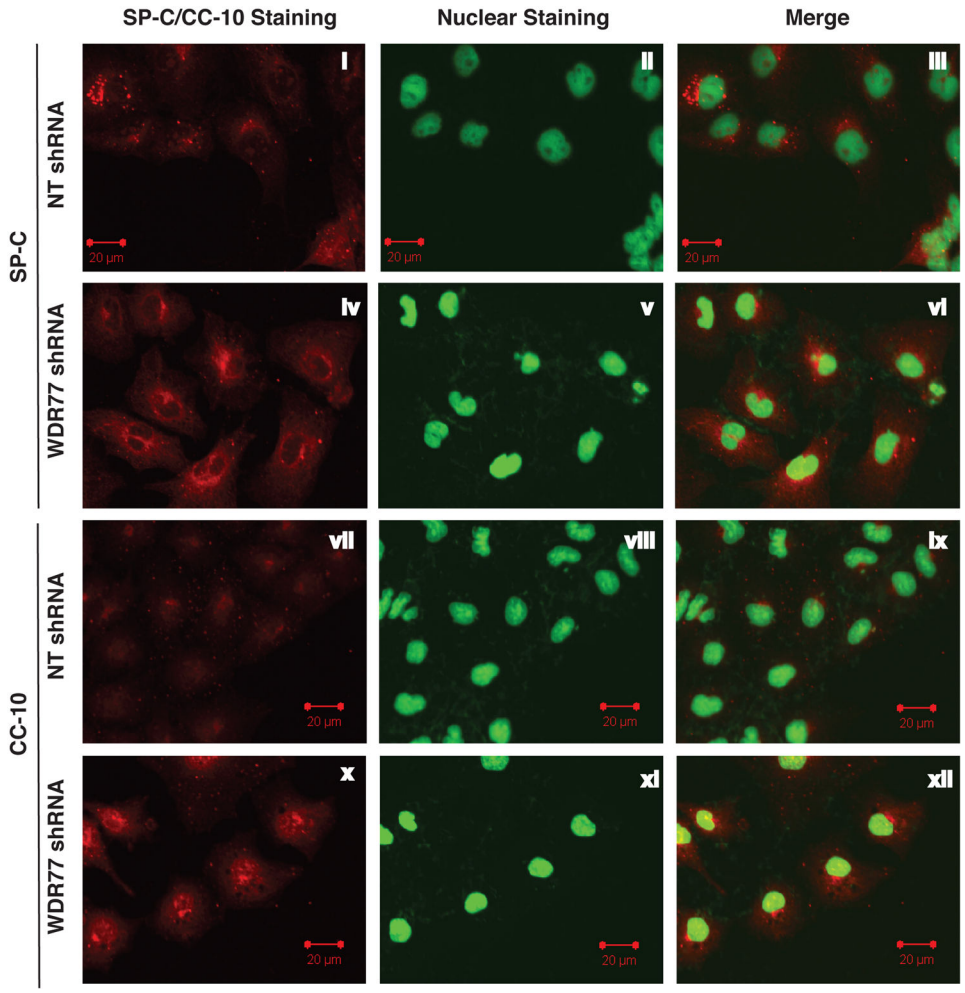

Figure 8.

WDR77 inhibited lung cancer cell differentiation. A549 cells expressing non-target (NT) shRNA or WDR77 shRNA were immunostained with anti-SP-C (I and IV) or -CC-10 (VII and X). Nuclei were stained with Sytox green (II, V, VIII, and XI).